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Killing of *dsrA* Mutants of *Haemophilus ducreyi* by Normal Human Serum Occurs via the Classical Complement Pathway and Is Initiated by Immunoglobulin M Binding

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Previously, we showed that serum resistance in *Haemophilus ducreyi* type strain 35000HP required expression of the outer membrane protein DsrA because the isogenic *dsrA* mutant FX517 is highly serum susceptible. In this study, we confirmed this finding by construction of additional serum-susceptible *dsr*A mutants in more recently isolated serum-resistant strains. We also demonstrated that killing of *dsrA* mutants required an intact classical complement cascade but not the alternative or mannan-binding lectin pathways. Between 5- and 10-fold more purified human immunoglobulin M (IgM) but not IgG was deposited onto *dsrA* mutant FX517 than onto parent strain 35000HP, consistent with IgM initiation of the classical cascade. Depletion of IgM, but not IgG, from complement-intact serum inhibited killing of FX517. As predicted from the amounts of IgM bound, more of the individual complement components were bound by FX517 than by parent strain 35000HP. Examination of the binding of negative regulators of complement as an explanation for serum resistance indicated that parent strain 35000HP bound more C4 binding protein and vitronectin than FX517 but not factor H. However, the degree and pattern of complement component binding observed suggested that IgM binding to the serum-susceptible mutant FX517 was responsible for the activation of the classical pathway and the observed killing of FX517 as opposed to binding of negative regulators of complement by the serum-resistant parent. We speculate that an undefined neo-epitope, possibly carbohydrate, is exposed in the *dsrA* mutant that is recognized by naturally occurring bactericidal IgM antibodies present in human sera.

Haemophilus ducreyi is the etiologic agent of chancroid, one of the sexually transmitted genital ulcer diseases. *H. ducreyi* is a fastidious gram-negative bacterium noted for its obligate requirement for heme and is a strict human pathogen. Chancroid is prevalent in many developing countries, including certain parts of Africa, Asia, and South America (28). There has been a renewed interest in chancroid because it has been shown to be an independent risk factor for the transmission of human immunodeficiency virus (32). Elimination of chancroid is feasible for commercial sex workers and results in a significant decrease of chancroid in their male clients (37a). Elimination of chancroid could potentially slow human immunodeficiency virus transmission in Africa.

H. ducreyi is highly resistant to killing by fresh normal human serum (fNHS) (commonly termed "serum resistance") (31). Serum resistance has been demonstrated to be a critical factor for survival and establishment of disease in many bacterial systems (25). Microbes utilize multiple strategies to resist killing by fNHS. A common mechanism of serum resistance uses surface-exposed bacterial proteins to bind negative regulators of complement (C′). For example, certain *Neisseria gonorrhoeae* porins (33), *Bordetella pertussis* filamentous hemagglutinin (5), and *Streptococcus pyogenes* M proteins (6) bind C4 binding protein (C4bp). *Borrelia burgdorferi* OspE (18), *Neisseria gonorrhoeae* Por1A (35), and *Streptococcus pneumoniae* PspC (12) bind factor H (fH). It is not known what mechanism *H. ducreyi* uses to resist killing by fNHS.

Early studies on serum resistance in *H. ducreyi* by Odumeru and colleagues in the mid-1980s showed that virulent strains were resistant to fresh normal human or rabbit serum while avirulent strains were serum susceptible (31). In Odumeru's studies, serum-susceptible strains contained truncated lipooligosaccharide (LOS) and were nonisogenic to the serum-resistant strains; Odumeru concluded that truncated LOS was responsible for the serum-susceptible phenotype (29–31). However, two more recent studies using isogenic mutants concluded that LOS (15, 19) is not a major determinant of serum resistance in *H. ducreyi*. LOS isogenic mutants of type strain 35000HP are not serum susceptible (19) and are fully virulent in the human model of chancroid infection (44, 45). In contrast, isogenic or naturally occurring mutants in the gene encoding the outer membrane protein DsrA are 10- to 100-fold more serum sensitive than virulent parent strain 35000HP. The isogenic *dsrA* mutant FX517 was attenuated in the human model of chancroid infection (7), emphasizing the importance of DsrA. Very recently, we have identified a second novel outer membrane protein also required for expression of full serum resistance in *H. ducreyi* (22). *dltA* mutants are moderately serum susceptible, and *dltA*/*dsrA* double mutants are the most serum susceptible of any *H. ducreyi* strain reported to date.

Odumeru's studies also examined the role of the classical

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and phenotype	Source or reference; place of isolation
Strains		
H. ducreyi		
35000HP	Wild type, parent	37
FX517	35000HP *dsrA*, Cm^r	15
425	Wild type, parent	13; Mississippi
FX529	425 *dsrA*, Cm^r	This work
010-2	Wild type, parent	13; Dominican Republic
FX530	010-2 *dsrA*, Cm^r	This work
E. coli DH5α	*recA gryB*	Invitrogen
Plasmids		
pUNCH 1256	Mutagenized *dsrA* in pRSM1791	15
pUNCH 1260	*dsrA* PCR clone in pLSKS	15
pLSKS	Shuttle plasmid, Str^r	43

and alternative pathways in serum resistance and concluded that killing of serum-susceptible *H. ducreyi* was due to the classical pathway, since EGTA inhibited killing. However, this result, observed prior to the recognition of the importance of the mannose binding lectin (MBL) pathway, would also be consistent with activation by the MBL pathway. Furthermore, results from studies on serum resistance and *H. ducreyi* conducted by Lagergard et al. (21) suggested that in addition to the classical pathway, the alternative pathway might also be important in the killing of *H. ducreyi.*

The objective of this study was to confirm and extend our earlier finding that DsrA expression is required for serum resistance by using more recently isolated and geographically distinct *H. ducreyi* strains and their isogenic *dsrA* mutants. Furthermore, we sought to clarify the relative roles of the classical, MBL, and alternative complement pathways in the killing of serum-susceptible *H. ducreyi*. Finally, we sought to identify where in the complement cascade activation occurred.

MATERIALS AND METHODS

Strains and media. The *H. ducreyi* strains used in this study are shown in Table 1. The extensively characterized type strain 35000HP and two recent isolates, 010-2 and 425 (13), and their corresponding *dsrA* mutants, FX517 (15), FX529, and FX530, were grown on chocolate agar plates (GC medium base, 1% hemoglobin, 1% GGC) supplemented with 5% fetal calf serum (FCS). Optimal growth and a more consistent expression of the serum-resistant phenotype was obtained when strains were grown on chocolate agar containing 5% FCS than in previous studies where FCS was not used (data not shown). Strains were maintained at 35°C with 5% CO_2.

Construction of *dsrA* mutants in recent isolates. *dsrA* mutants for parent strains 010-2 and 425 were constructed as previously described for strain 35000HP (15). The mutagenesis plasmid pUNCH 1256 (15) was used to electroporate 010-02 and 425 to form FX529 and FX530, respectively. Strains were confirmed by PCR using *dsrA* primers 14 and 24 (15). PCR of mutants yielded a band approximately 1 kb larger than that of the parent, consistent with the insertion of a 1-kb chloramphenicol cassette in the *dsrA* gene. Western blotting using antiserum to recombinant DsrA (9) revealed that each mutant lacked expression of DsrA.

Complementation of *dsrA* mutants FX529 and FX530. The *dsrA* mutants FX529 and FX530 were complemented as previously described by Elkins et al. (15). Briefly, plasmid pUNCH 1260 *dsrA* (strain 35000HP) or the empty vector pLSKS (negative control) were electroporated into *dsrA* mutants FX529 and FX530. Strains containing pUNCH 1260 or pLSKS were grown on chocolate agar containing streptomycin at 100 μg/ml. Expression of *dsrA* in complemented mutants was confirmed by Western blotting. Bactericidal assays were performed using the complemented mutants as described below.

Bactericidal assay. Bactericidal assays were performed as previously described (15). Chocolate agar cultures of *H. ducreyi*, 16 to 18 h old, were used to prepare bacterial suspensions for each strain at an optical density at 600 nm (OD_{600}) of 0.2 (approximately 1×10^8 CFU/ml) in gonococcal medium base broth (GCB) (Difco). Fifty microliters of a 10^{-5} dilution of *H. ducreyi* was mixed with 50 μl of either pooled fNHS or heat-inactivated NHS (hNHS) to achieve a final concentration of 50% serum. Normal human serum was prepared from blood collected from four volunteers. In experiments using various percentages of sera, volumes of bacteria and serum were adjusted appropriately to the desired concentration of serum. Following incubation for 45 min at 35°C in the presence of 5% CO_2, aliquots were plated onto chocolate agar plates and incubated for 48 h, and viable colonies were counted. Data are expressed as percent survival in fNHS compared to that in hNHS [(CFU in fNHS/CFU in hNHS) × 100].

Inhibition of classical and MBL pathways. Activation of the complement system proceeds by three pathways, the classical pathway, the MBL pathway, and the alternative pathway (41). To determine which pathway is responsible for killing in *H. ducreyi*, we used EGTA to chelate the calcium ions in NHS. The classical pathway and the MBL pathway are both calcium-dependent pathways and are therefore inactive in calcium-depleted serum (11, 26, 38, 39). The alternative pathway requires magnesium for its activity and is not affected by the absence of calcium. However, because EGTA also binds magnesium, albeit at a lower affinity, we supplemented the chelated serum with 2 mM $MgCl_2$ (16). Fifty microliters of NHS, chelated with 20 mM EGTA plus 2 mM $MgCl_2$ (fNHS-EGTA/$MgCl_2$), was added to 50-μl aliquots of *H. ducreyi*, and the bactericidal assay was performed as described above.

Specific inhibition of classical pathway. In order to differentiate between the role of the classical pathway and that of the MBL pathway in killing of *H. ducreyi*, we used C1q-depleted serum (Advanced Research Technologies [ART]). Removal of C1q from normal human serum specifically inhibits the classical pathway but not the MBL pathway (26). Fifty-microliter aliquots of *H. ducreyi* suspensions were incubated with either 50 μl of fNHS, C1q-depleted fNHS (fNHS/C1q−), or C1q-depleted serum reconstituted with 100 μg/ml of purified C1q (fNHS/C1q−/C1q+), and the bactericidal assay was performed as previously described. The process used to deplete the serum of C1q also chelates the calcium and magnesium ions (William Kolb, ART, personal communication). Therefore, we supplemented our C1q-depleted serum with 5 mM $CaCl_2$ and 2 mM $MgCl_2$ to restore classical pathway activity. The level of MBL in the Clq-depleted serum was examined by Western blotting and found to be comparable to that of NHS (data not shown).

Statistical analysis. Comparison of serum-resistant parents and serum-susceptible *dsrA* mutants was performed using a standardized *t* test for multiple comparisons. A P value of ≤ 0.05 was accepted as the level of statistical significance.

Immunoglobulin binding to *H. ducreyi*. Two hundred micrograms of purified human immunoglobulin M (IgM) (Sigma I-8260, lots 115H48011 and 087H4832) or IgG (purified from NHS using protein G agarose [Sigma]) was iodinated as previously described (15). Fifty microliters of *H. ducreyi* 35000HP, FX517 (OD = 0.4, approximately 2×10^8 CFU), or no bacteria suspended in GCB was mixed with ^{125}I-IgM or IgG (2.5×10^6 cpm) in a multiscreen plate (Multiscreen-HV, catalog no. MAHVN4550; Millipore, Inc). Plates were incubated at 34°C in 5% CO_2 for 45 min. Plates were suctioned and washed five times with phosphate-buffered saline (PBS), and *H. ducreyi*-associated cpm were determined by gamma counting. Net cpm bound to 35000HP or FX517 were determined by subtracting cpm present in wells that received no *H. ducreyi*.

Western blot assays for the measurement of complement components. Complement protein deposition onto *H. ducreyi* was measured following incubation with NHS. Bound complement components were measured by Western blotting using specific antisera or monoclonal antibodies (MAbs) to various complement proteins. *H. ducreyi* suspensions were prepared in GCB to an OD of 0.5 at 600 nm using a 16- to 18-h culture of 35000HP or FX517. Bacterial suspensions (500 μl) were mixed with indicated volumes of either fNHS or hNHS to achieve the desired percentage of NHS and incubated for 45 min. For time course studies, samples were incubated for 1, 10, 30, or 45 min. To verify that bands observed in Western blots were not due to antibody binding to *H. ducreyi* proteins, bacterial suspensions were incubated in the absence of NHS. Following incubation, cells were placed on ice for 5 min, centrifuged for 2 min, and washed three times with cold GCB. After the final wash, cell pellets were resuspended in 100 μl of Laemmli sample buffer. Twenty-microliter samples were electrophoresed on a sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and electrotransferred to a nitrocellulose membrane. Samples for C4bp, C6, and C7 detection were prepared under nonreducing conditions, because under reducing conditions no signal was obtained. All other gels were run under reducing conditions. Mem-

branes were blocked with 2% bovine serum albumin in 1× PBS containing 0.1% azide (blocker) for 1 h. Membranes were then probed with the following complement antibodies: C7 and C6 (ART) and C4bp (Quidel) at a 1:5,000 dilution; C3 and C4 (ART) at a 1:1,000 dilution in blocker. The secondary antibodies used were anti-mouse or anti-goat alkaline phosphatase (Sigma) as appropriate at a 1:5,000 dilution. Bound antibody was detected using a chemiluminescent substrate (Lumi-Phos; Pierce).

Well-characterized *Neisseria gonorrhoeae* strains FA6564 (Por1A) and F62 were used as controls to test for the ability of our Western blot assay to discriminate between differences in complement protein deposition (34). FA6564 is resistant to killing in 10% NHS, whereas F62 is susceptible. Gonococcal strains were incubated with NHS as described above, using a final serum concentration of 10%. Western blot analysis of complement binding to these strains was carried out as described for *H. ducreyi* strains.

C9 deposition. C9 deposition was determined using ^{125}I-labeled anti-SC5b-9 monoclonal antibody (Quidel, catalog no. A239). One hundred micrograms of antibody was iodinated as previously described (14). *H. ducreyi* cell suspensions were prepared to an OD_{600} of 0.5 in GCB medium, and 100 μl of this suspension was mixed with 100 μl of either GCB, hNHS, or fNHS in a 96-well multiscreen plate. Following incubation with NHS, cells were suctioned onto the filter membrane and washed four times with 1× PBS. Approximately 1×10^6 cpm of iodinated anti-SC5b-9 was added to each well, and binding was allowed to proceed for 30 min at room temperature. Cells were again washed four times with 1× PBS-Ca-Mg, and filters containing the bacteria and bound proteins were allowed to dry. Dried filters were punched out into 12- by 75-mm glass tubes, and *H. ducreyi*-associated cpm were determined using a gamma counter.

C4BP binding to *H. ducreyi*. Purified C4BP (generously provided by Anna Blom, University of Lund, Malmo, Sweden) binding was performed as described above for purified immunoglobulin binding, except that iodinated C4BP was used. Iodinated C4BP (approximately 1×10^6 cpm) was mixed with *H. ducreyi* or gonococcal controls (approximately 1×10^7 CFU in a total volume of 100 μl) for 45 min. After washing bacteria, bound C4BP was determined by gamma counting.

C-reactive protein (CRP) and phosphorylcholine (PC) experiments. Immobilized PC (Pierce Chemicals) was used to deplete CRP from NHS using PC agarose (Pierce Chemicals) (42). Greater than 95% of CRP was depleted as assayed by Western blotting using anti-CRP (data not shown). As an additional control, *Haemophilus influenzae* strains expressing (strain 418) or not expressing (strain 419) PC on their LOS were used as controls (17). Bactericidals of *H. influenzae* utilized 10% NHS, whereas bactericidals of FX517 utilized 20% serum. We also examined whether *H. ducreyi* expressed PC on its LOS by Western blots using the anti-PC MAb TEPC-15 and used *H. influenzae* strains 418 and 419 as positive and negative controls, respectively (42). Crude LOS was prepared by using proteinase K as described by Hitchcock and Brown (20).

Depletion of immunoglobulins. IgG was depleted from fresh NHS by using recombinant protein G agarose (no ×1197; capacity, 20 mg/ml human IgG of packed gel; exalpha). IgM was depleted using anti-human IgM agarose (no. A-9935; capacity, 2.4 mg/ml packed gel; Sigma). Briefly, an excess of prechilled immunosorbent (2 ml packed) equilibrated in cold PBS was mixed with chilled fNHS (3 ml, pooled or individual sera) and gently rocked for 15 min on ice. The agarose was centrifuged, and the serum supernatant was filter sterilized. This process diluted NHS approximately by 1/3 based on total protein determinations due to residual PBS in packed agarose. Control agarose lacking bound ligands was used to control for nonspecific binding of serum components and serum dilution. The depleted sera were tested for bactericidal activity against strain FX517 as described above. Depletion of IgG and IgM was confirmed at the UNC Hospitals Clinical Immunology Laboratories and in Western blots in our laboratory using antisera specific to each class. Greater than 90% of IgG or IgM was removed by the adsorption techniques described above.

To confirm that depletion of IgM inhibited killing of FX517, we reconstituted depleted serum with purified IgM (I-8260; Sigma). IgM-depleted serum when reconstituted with purified IgM brought IgM levels to normal serum levels. To ensure that depletion of IgM had no effect on IgG levels or activity, we used an additional control in which we added IgG (I-4506; Sigma; or eluted from the protein G column above) to IgM-depleted sera. IgM-depleted serum reconstituted with purified IgG resulted in levels of IgG that were approximately twice that of NHS. The intact complement activity was also measured by the addition of heat-inactivated serum (as the source of immunoglobulins) to IgM-depleted sera. The reconstituted sera were used in bactericidal assays with FX517.

Absorption of fNHS with FX517 to remove bactericidal antibodies. Strain FX517 was suspended in GCB medium to an OD_{600} of 1.0. This suspension was chilled, 1 ml was centrifuged, and the pellet was resuspended in 0.4 ml fNHS. After incubation for 1 h, the suspension was centrifuged and the supernatant absorbed twice more but for 30 min each. After the last absorption, the serum was filter sterilized (0.45 μm) and used for bactericidal assays as described above at 25%. Absorbed serum bactericidal activity was reconstituted with purified IgM as described in the preceding section or with 25% hNHS (as a source of immunoglobulins).

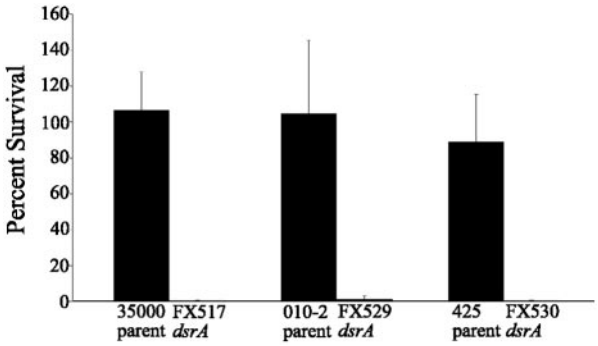

FIG. 1. Bactericidal killing of *H. ducreyi* parent strains and *dsrA* mutants. The indicated parent strains and their isogenic *dsrA* mutants were incubated with NHS for 45 min, and numbers of CFU were determined by plating. Percent survival was calculated by dividing the CFU from fNHS by CFU from hNHS and multiplying by 100. The *P* values for strains 35000HP (35000), 010-2, and 425 and each of their isogenic mutants were <0.0001, <0.0001, and <0.0001, respectively.

RESULTS

Further evidence that expression of *dsrA* is required for serum resistance in *H. ducreyi*. Previous work from our laboratory showed that DsrA is required for high-level serum resistance in *H. ducreyi* strain 35000HP and in certain other strains isolated in the distant past (15). To confirm the importance of DsrA in serum resistance, we constructed isogenic *dsrA* mutants in more recently isolated strains. Strains 010-2 and 425 were chosen for *dsrA* mutagenesis based on the following criteria: high levels of serum resistance, geographic distinction, and lack of plasmids and chloramphenicol sensitivity. *dsrA* mutants FX529 (parent strain 010-2) and FX530 (parent strain 425) were confirmed by PCR and Western blotting as described in Materials and Methods. In contrast to parent strains 010-2 and 425, which exhibited high levels of serum resistance, FX529 and FX530 were more serum susceptible, exhibiting only 1% and 0.1% survival (Fig. 1). Complementation of FX529 and FX530 with a plasmid expressing DsrA from strain 35000HP (pUNCH 1260) restored the serum-resistant phenotype (data not shown). These results confirm our earlier findings and provide further evidence for the essential role of DsrA in serum resistance.

The classical pathway is required for complement-mediated killing of *H. ducreyi* mutant FX517. Activation of complement generally requires antibody binding (classical pathway), mannan binding lectin binding to carbohydrate structures on microbes (MBL pathway), or direct C3b binding to microbial surfaces (alternative pathway) (41). EGTA, in the presence of Mg^{2+} (EGTA/Mg), inhibits both the classical and the mannose binding lectin pathway but not the alternative pathway in bactericidal killing assays (26). Treatment of serum with EGTA/Mg prevented killing of serum-susceptible *dsrA* mutants FX517 and FX529 (88% and 100% survival, respectively [Fig. 2]) as contrasted to nonchelated serum (0.1 and 0.8 percent in fNHS for FX517 and FX529, respectively). To distinguish between the classical and MBL pathways, we conducted

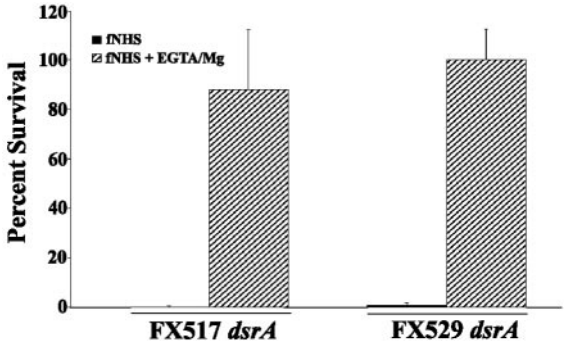

FIG. 2. NHS killing of *dsrA* mutants requires Ca^{2+}. The indicated *dsrA* mutants were subjected to bactericidal killing in the presence and absence of EGTA/Mg. *P* values for FX517 and FX529 (fNHS versus fNHS plus EGTA/Mg) were <0.0001 for both strains.

bactericidal experiments using C1q-depleted serum, a C′ component required for the classical pathway but not required for the MBL pathway. The survival rates for the serum-susceptible *dsrA* mutants FX517 and FX529 in fNHS were 1.2% and 1%, respectively (Fig. 3). When FX517 and FX529 were incubated with C1q-depleted NHS, the survival rate increased to 93% and 88% survival, respectively. The addition of purified C1q to the C1q-depleted serum partially restored serum killing (21% and 20% survival for FX517 and FX529, respectively). We did not fully reconstitute killing. However, in erythrocyte lysis assays (50% hemolytic complement assay) used to determine the efficiency of reconstituted C1q serum, the complement activity is at least 80% of that in normal serum (personal communication; Kolb). This 50% hemolytic complement value is comparable to the level of bacterial killing observed in our study. To further examine the possible role of MBL activation of complement, we measured MBL binding. Less than 0.5 nanograms of MBL bound to the surfaces of 35000HP and FX517 (data not shown). These data suggest that the classical but not the mannan binding lectin or alternative pathway is essential for killing of *dsrA* mutants FX517 and FX529.

Immunoglobulin binding to *H. ducreyi*. Generally, activation of the classical pathway is achieved through antibody binding,

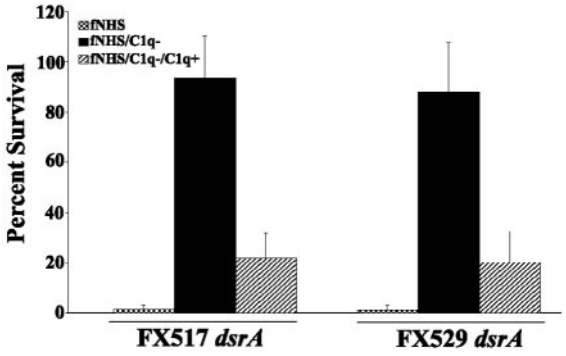

FIG. 3. Bactericidal killing of *dsrA* mutants requires Clq. Bactericidal killing (50% NHS) was performed as described using NHS (fNHS), NHS depleted of C1q (fNHS/C1q×), or NHS depleted of C1q and then reconstituted with physiological concentrations of purified C1q (fNHS/C1q−/C1q+). *P* values for FX517 and FX529 (fNHS versus fNHS/C1q−) were < 0.0001 and < 0.0001, respectively. *P* values for both strains (fNHS/C1q− versus. fNHS/C1q−/C1q+) were <0.0001.

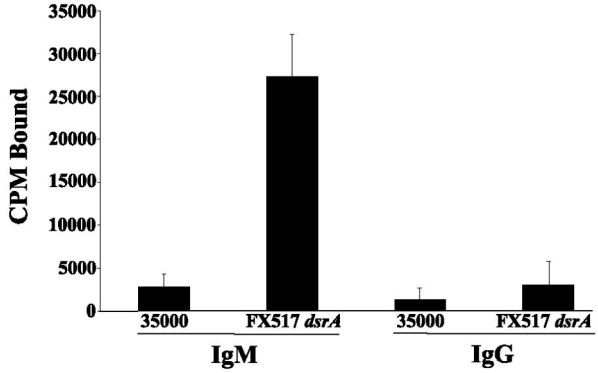

FIG. 4. Binding of immunoglobulins by *H. ducreyi*. The indicated strains of *H. ducreyi* (1×10^7 CFU) were mixed with iodinated purified human IgM or IgG (1×10^6 cpm) in a total volume of 100 μl. After 45 min, the *H. ducreyi* strains were suctioned and washed five times with PBS, and bacteria-associated radioactivity was determined. *P* values for 35000HP (35000) versus FX517 for IgM depletion and IgG depletion were <0.0001 and <0.0693, respectively.

although in certain systems, spontaneous binding of C′ components in the absence of antibody occurs, leading to bacterial death (1, 2, 42). To measure immunoglobulin-mediated initiation of the classical pathway, iodinated purified human IgM or IgG was mixed separately with suspensions of *H. ducreyi* 35000HP and FX517. After incubation and washing, the amount of bound immunoglobulin was determined by gamma counting. *H. ducreyi* FX517 bound approximately 5- to 10-fold more IgM than parent strain 35000HP, depending on the lot of IgM used (Fig. 4). The difference in IgG binding to 35000HP and FX17 was not as great as that observed for IgM binding and did not reach statistical significance.

C1q binding. C1q, the first C′ component, was studied using a time course experiment. Using fNHS as the source of C1q, C1q was deposited as early as 1 min on FX517 (Fig. 5). Interestingly, the amount of C1q deposited in fNHS increased and then decreased over time. Clq deposition was not observed in 35000HP samples incubated in fNHS. In hNHS, where C1q is denatured and inactive (26), C1q binding was observed only in samples incubated for 45 min with hNHS. Using purified C1q or C1qrs holocomplex in the absence of antibody, more C1q was deposited at 45 min on FX517 than on 35000HP (data not shown). Taken together, C1q binding in heated NHS at later time points and purified C1q binding may represent nonfunctional binding, since this binding neither correlated temporally with the binding of C1q nor with the killing of bacteria in fNHS (data not shown).

C4 and C3 binding. C4 and C3, the components immediately following C1, were measured using the Western blot assay. Functional C4 and C3 alpha chains, but not beta chains, form covalent ester and amide bonds with certain acceptor molecules on the membranes of bacteria that are not broken by the conditions used for SDS-PAGE in this study (26). Since the various acceptor molecules vary in molecular mass, the alpha chain complexes often form a heterogeneous smear of covalently bound reactive material in the fNHS lanes. The beta chains released from these complexes migrate independently of the alpha chains and acceptor molecules. For all forms of C4 and C3, more was deposited on FX517 than on 35000HP (Fig.

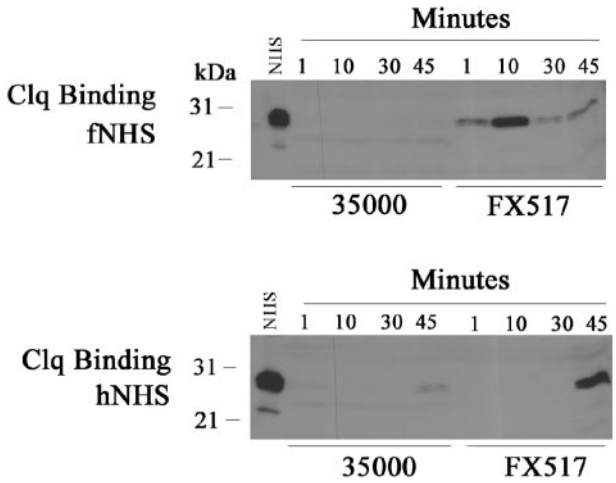

FIG. 5. C1q binding to *H. ducreyi* as measured by immunoblotting. *H. ducreyi* strains were incubated with fNHS for the indicated time periods. After binding of complement components, bacteria were pelleted and washed by cycles of centrifugation. Cell pellets with bound complement components were subjected to SDS-PAGE and immunoblotting using polyclonal anti-human C1q. C1q is composed of three individual chains, A, B, and C, that migrate at 27.5, 25.2 (doublet), and 23.8 kDa, respectively. Similar results were observed in three other experiments. Normal human serum, 0.1 μl, was loaded in the first lane as a positive control (approximately 7 nanograms of C1q).

6, right panel). Specifically, high-molecular-weight smears of immunoreactive material representing C4 and C3 alpha chains covalently bound to acceptor molecules were observed in fresh but not heated NHS. Similarly, there was an increase in noncovalently bound beta chains in FX517 incubated in fNHS compared to results with hNHS. Noncovalent binding of C3 was observed in hNHS samples and migrated at a position in the gel consistent with the reduced forms of C3 alpha chain

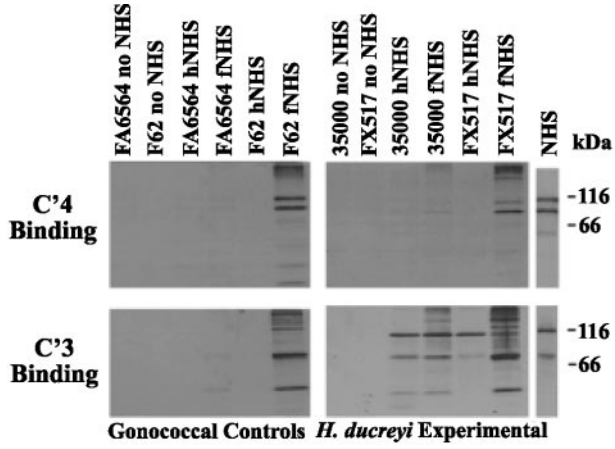

FIG. 6. Binding of C3 and C4 to *H. ducreyi.* Bacteria were incubated in the absence of NHS or in the presence of hNHS or fNHS. After washing bacteria to remove unbound serum proteins, bound C′ proteins were detected by SDS-PAGE (reducing conditions) using anti-human C3 and C4. NHS, 0.1 μl, was loaded as a positive control. The upper band in the NHS standard lane is the alpha chain, and the lower band is the beta chain for both C4 and C3. FA6564 (serum resistant) and F62 (serum susceptible) are gonococcal control strains. Similar results were obtained in three other experiments.

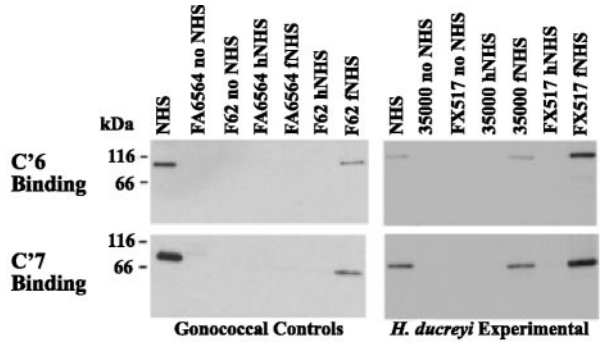

FIG. 7. Binding of C6 and C7 by *H. ducreyi*. Bacteria were incubated in the absence of NHS or in the presence of hNHS or fNHS. After unbound proteins from bacteria were washed, bound proteins were detected using anti-human C6 or C7 in Western blots. Similar results were observed in three other experiments.

(115 kDa) and beta chain (75 kDa) (Fig. 6, hNHS lanes). Time course experiments indicated that C4 and C3 binding to FX517, like that of C1q, was rapid, with deposition occurring as early as 1 min postincubation (data not shown). In these studies we used well-characterized gonococcal strains that are serum resistant (FA6564) and serum susceptible (F62) in order to evaluate whether our methods could discriminate between bacteria that bind low or high amounts of C′4 and C′3, respectively (Fig. 6, left panel).

Terminal MAC binding. Western blots were also used to detect the noncovalent binding of complement proteins C5b through C8. In experiments measuring C6 and C7 binding to *H. ducreyi* strains, more of each was deposited on FX517 than on 35000HP (Fig. 7, right panel). Control experiments using gonococcal strains revealed more C6 and C7 binding to F62 (serum sensitive) than to FA6564 (serum resistant) (Fig. 7, left panel). In C9 binding experiments, more C9 was deposited on serum-susceptible FX517 than on serum-resistant 35000HP (15,000 cpm and 3,400 cpm, respectively) (Fig. 8). Taken together, these results show that more terminal membrane attack complex (MAC) was deposited on serum-susceptible FX517

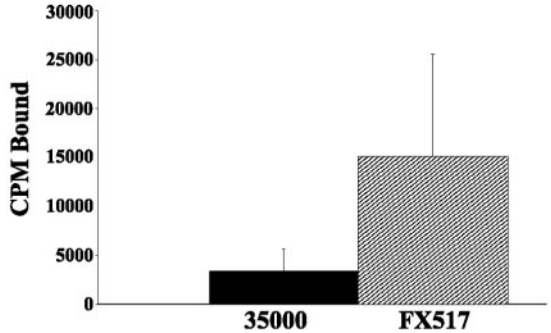

FIG. 8. Binding of terminal MAC complex. *H. ducreyi* strains were incubated in fNHS or hNHS (10%) for 45 min at 35°C. Bacteria were suctioned and washed with PBS, and an iodinated MAb directed against a C5b-C9 neoepitope was added (1×10^6 cpm). After binding of MAb and removal of unbound MAb by washing, bacterium-associated cpm were determined. Shown are the cpm bound in fNHS minus the cpm bound in hNHS. The *P* value for 35000HP (35000) versus FX517 was <0.0004.

than on serum-resistant 35000HP, consistent with the previous results from bactericidal assays.

Negative regulators of complement binding to *H. ducreyi*. Some serum-resistant bacteria avoid killing by NHS by directly binding negative regulators of complement (without a requirement for other functional complement components). Recently, we demonstrated that *H. ducreyi* DsrA directly binds the negative complement regulator vitronectin (9). It has been proposed that vitronectin may play a role in mediating resistance in other bacteria (24, 36) by preventing the deposition of terminal complement components (C5b through C9). If DsrA-bound vitronectin was the sole factor and acted as a negative complement regulator in *H. ducreyi*, the deposition levels of the earlier complement components (antibody, C1q through C3) would be expected to be similar for 35000HP and FX517, whereas deposition of the terminal MAC components (C5b through C9) would be greater on serum-susceptible FX517. This is not what we observed in the experiments described above.

We also measured the binding of other negative regulators of C′ to *H. ducreyi* to determine if this could account for its serum resistance phenotype. fH is a cofactor for factor I (fI)-mediated cleavage of the alternative pathway C3 convertase (C3bBb). In addition to inhibiting alternative pathway activation, direct fH binding by bacteria also prevents the alternative pathway amplification of an IgM-initiated complement cascade (27). We used heated sera as a source of fH and C4bp (see below), which are stable with heat treatment (27). hNHS was mixed with *H. ducreyi*, and fH binding was measured in Western blots using anti-fH. fH was minimally bound by *H. ducreyi* strains 35000HP and FX517; both bound less than 50 ng of fH per 10^7 CFU (data not shown). Similar results were seen when purified fH was used in binding experiments (data not shown).

Previously, it was shown that stable serum resistance in gonococcal Por1A strains, including FA6564, was due to the binding of C4bp, a cofactor for fI (34). C4bp binding causes the dissociation of the C3 convertase and also promotes the cleavage of C4b into the inactive fragments C4c and C4d. We measured C4bp binding to bacteria using two methods. In the first method, iodinated purified C4bp was mixed with bacteria, and bound C4bp was measured by gamma counting of washed bacteria. In experiments using iodinated C4bp and gonococcal controls, the serum-resistant positive control strain, FA6564, bound a mean of 578,759 ± 49,121 cpm. The serum-susceptible gonococcal negative control strain F62 bound 568 ± 206 cpm of C4bp. In *H. ducreyi* experimental samples, serum-resistant *H. ducreyi* strain 35000HP bound a mean of 9,396 ± 1,417 cpm, and serum-susceptible *H. ducreyi* strain FX517 bound 3,567 ± 34 cpm. In the second method of C4bp binding, hNHS was used as the source of C4bp, and binding was detected by Western blotting. In these experiments, slightly more C4bp was associated with 35000HP than with FX517 (data not shown). Both methods of C4bp binding revealed that between 10- and 60-fold more C4bp was associated with serum-resistant gonococcal strain FA6564 than with serum-resistant *H. ducreyi* 35000HP; thus, 35000HP bound relatively minor amounts of C4bp.

CRP is known to bind PC (40). Bacterium-bound CRP is capable of directly activating the classical pathway of complement by binding C1q without a need for immunoglobulins. Certain nontypeable *H. influenzae* strains express PC on their LOS, which binds CRP present in NHS. These *H. influenzae* strains are killed by the C′ cascade in an immunoglobulin-independent manner. If *H. ducreyi* strain FX517 expressed PC, it was possible that PC-mediated C1q binding, as opposed to IgM, initiated the complement cascade. To examine this issue, we removed CRP from serum and tested its ability to kill *H. ducreyi* FX517 as well as the *H. influenzae* control strain 418, which expresses PC. After CRP depletion, FX517 was killed in a manner similar to that with CRP-replete serum (data not shown); however, *H. influenzae* strain 418 was no longer killed. Furthermore, using TEPC-15, a MAb that recognizes PC, no reactivity was observed in Western blots of *H. ducreyi LOS* or total cellular proteins. PC-positive LOS, but not PC-negative LOS, from *H. influenzae* control strains was recognized by TEPC-15 (data not shown). These data suggest that PC-mediated CRP binding cannot account for the killing of FX517.

Depletion of IgM inhibits killing of FX517. In order to demonstrate a cause and effect relationship between IgM binding and killing of *H. ducreyi* strain FX517, we depleted immunoglobulin (IgM or IgG) from human NHS. In these studies we chose to use a reduced serum concentration (20%) for technical reasons. First, 20% serum approaches the lowest concentration of serum that effectively kills FX517 (15). Leduc (22) recently showed that 10% NHS results in about 69% survival, whereas 25% NHS kills 99% or more (15). Furthermore, removing IgM (1 mg/ml) and IgG (10 mg/ml) from serum while maintaining intact complement is technically demanding, and we could conserve reagent sera by using 20% NHS instead of 50%.

Anti-human IgM agarose was briefly incubated with NHS on ice to deplete the IgM and to preserve complement activity. FX517 incubated in 20% fNHS, without the dilution effect of agarose treatment, had a survival rate of 19% (Fig. 9). Mock treatment of fNHS with agarose containing no ligand resulted in 37% survival for FX517, presumably due to dilution effect (~1/3 dilution). In contrast, treatment of serum with agarose containing covalently bound anti-human IgM (IgM-depleted serum) resulted in a 71% survival rate. The killing activity of IgM-depleted NHS was restored when purified IgM was reconstituted to normal serum concentrations (30% survival for FX517). This level of survival is similar to the level obtained for mock IgM-depleted sera, which are similarly diluted (37%). Purified IgG added to IgM-depleted serum served as an additional control to ensure that the depletion of IgM from fNHS did not adversely affect IgG levels or activity. The addition of IgG, to a twofold excess of IgG, to IgM-depleted serum did not restore killing (81% survival for FX517). The addition of heat-inactivated serum (as a source of immunoglobulins) to IgM-depleted serum restored killing activity, with FX517 exhibiting a survival rate of 32%. In experiments not presented here, removal of IgG from NHS using protein G agarose had no effect on the killing of FX517. The above data suggest that IgM, but not IgG, plays a critical role in NHS-mediated killing of FX517.

Absorption of NHS with FX517 removes bactericidal IgM. To confirm IgM was responsible for initiation of the complement cascade, we incubated *H. ducreyi* strain FX517 *dsrA* with NHS on ice to remove the antibodies responsible for killing (putatively IgM) but maintain complement activity. FX517 survival in absorbed fNHS was 54% compared to only 1.9% for unabsorbed fNHS (25% NHS without significant dilution) (Fig. 10). Killing activity against FX517 *dsrA* was restored upon addition of purified IgM or if heated NHS was added as a

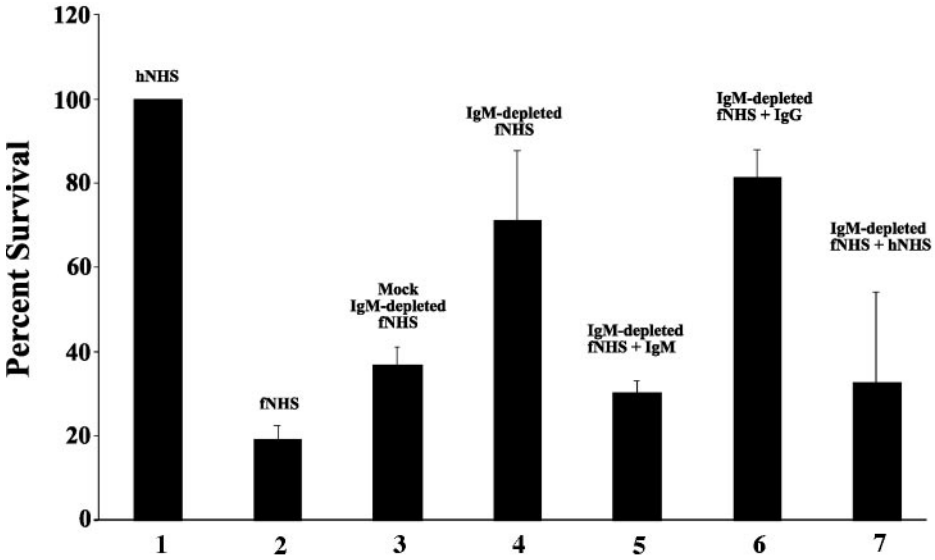

FIG. 9. Fresh normal human serum was treated as described here, and the bactericidal activity was determined using a 20% concentration. 1, fNHS heated to 56°C to inactivate the complement (hNHS); 2, fNHS incubated on ice without treatment (fNHS); 3, fNHS incubated with agarose containing no ligand on ice (mock IgM-depleted fNHS); 4, fNHS incubated with anti-human IgM agarose on ice (IgM-depleted fNHS); 5, IgM-depleted fNHS reconstituted with IgM (IgM-depleted fNHS + IgM); 6, IgM-depleted fNHS reconstituted with IgG (IgM-depleted fNHS + IgG); 7, IgM-depleted fNHS reconstituted with hNHS as a source of immunoglobulins (IgM-depleted fNHS + hNHS).

source of immunoglobulins, 4% and 5% survival, respectively. The survival rate of FX517 in hNHS supplemented with IgM was 90%, indicating that IgM-mediated killing required an intact complement cascade. These data provide further evidence that IgM is important in NHS-mediated killing.

DISCUSSION

Serum resistance. In order to initiate human infection, *H. ducreyi* requires hemoglobin as a source of heme (3, 23). During acquisition of heme from blood, *H. ducreyi* is exposed to human serum antibodies and complement. Thus, it is not too surprising that *H. ducreyi* has evolved mechanisms to avoid complement-mediated killing. The ability to avoid killing by NHS is an important virulence factor of bacterial pathogens, including *H. ducreyi*, since a *dsrA* mutant is highly attenuated (15).

Classical pathway is responsible for killing of FX517. In this paper, we present four lines of evidence that the classical pathway was responsible for killing of serum-susceptible strain FX517 by fNHS. (i) EGTA, which inhibits the classical pathway, inhibited killing. (ii) C1q, a C′ component unique to the classical pathway, was required for killing, and more C1q was bound by serum-susceptible FX517. (iii) Five- to 10-fold more IgM was bound to FX517 than to the parent strain, 35000HP. (iv) IgM was required for killing of FX517.

Prior to the discovery of the MBL pathway, Odumeru demonstrated that EGTA/Mg (30) abolished the killing of serum-susceptible *H. ducreyi* A77 (*dsrA* and *los* double mutant) and concluded the classical pathway was responsible for killing. EGTA/Mg also inhibits the MBL pathway; however, our evidence suggests that the MBL pathway is not involved, since MBL was not bound by *H. ducreyi* and C1q-depleted serum failed to kill FX517. Thus, we have confirmed and extended Odumeru's early studies. Lagergard et al. studied serum resistant *H. ducreyi* and found a minor role for the alternative pathway in the killing of *H. ducreyi* (21). We did not conduct specific experiments to assess the role of the alternative pathway in the killing of FX517. However, our data from studies of the classical and MBL pathways suggest that the alternative pathway does not play a major role. Furthermore, it must be noted that Lagergard's and our studies are not comparable because they used serum-resistant strains and NHS, immune serum, and rabbit complement. Our studies used serum-susceptible *H. ducreyi* and NHS as a source of complement.

Antibody and complement deposition on *H. ducreyi*. More IgM was deposited on FX517 than on 35000HP, and removal of IgM prevented killing. Based on these data, we propose that this event leads to complement activation on FX517. Two possibilities may explain why more IgM was deposited on FX517. Perhaps in the parent, DsrA physically shields another surface component(s), possibly LOS, to which bactericidal IgM antibodies are directed. Alternatively, perhaps a novel undetected antigen containing a bactericidal epitope(s) is expressed in the *dsrA* mutant compared to parent. Of the complement components we measured, more complement (from C1q through C9) was deposited on serum-susceptible strain FX517 than on serum-resistant strain 35000HP. For C4 and C3, the kinetics of deposition was more rapid on FX517 than on 35000HP. Finally, perhaps the ability of the C′ cascade to be deposited on 35000HP is prevented by DsrA.

The epitope for bactericidal IgM remains unknown. Naturally occurring antibodies to LOS are found in NHS and are often of the IgM isotype. For example, IgM anti-LOS antibodies are responsible for the initiation of the classical C′ cascade and subsequent killing of serum-susceptible gonococci (10). Serum-susceptible gonococci grown in the presence of exogenously supplied 5′cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA) sialylate their LOS and are rendered serum resistant. Serum-sensitive gonococci grown in media containing CMP-NANA

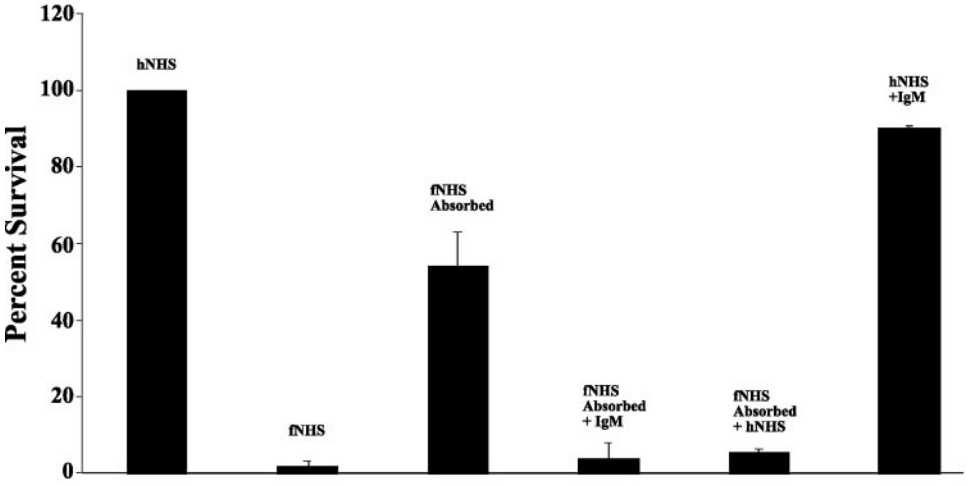

FIG. 10. Absorption of NHS with FX517 *dsrA* removes bactericidal IgM. fNHS was absorbed with FX517 *dsrA* on ice as described in the text. After absorption, fNHS was used in bactericidal assays against FX517. To determine if killing could be restored to absorbed serum, purified IgM equal to the amount present in NHS was added. Alternatively, hNHS was added as a source of immunoglobulins.

sialylate their LOS and become serum resistant. *H. ducreyi* are able to sialylate their LOS from endogenously synthesized CMP-NANA pools. Nevertheless, *H. ducreyi* mutants unable to sialylate their LOS remain fully serum resistant. Given the immunochemical similarities of the LOS of gonococci and *H. ducreyi*, these findings are somewhat surprising.

Yet another unusual phenomenon is that the highly truncated *H. ducreyi* LOS mutants 35000HP *glu* (44) and *gmhA* (4) are serum resistant (I. Nepluev and C. Elkins, unpublished data). In many bacterial systems, LOS mutants are both serum susceptible and avirulent. Human experimental chancroid challenge experiments using 35000HP *glu* revealed that this mutant retains virulence, consistent with the notion that serum resistance is required for infectivity in humans (44). LOS mutants have reportedly shown decreased attachment to keratinocytes (8), yet apparently this had no significant effect on virulence. We think it possible that LOS could be the target of the bactericidal IgM antibodies described in this paper. Experiments are in progress to identify the epitope(s) for bactericidal IgM.

We entertained the possibility that IgM was not involved in the killing of FX517 and that an antibody-independent C1q-dependent classical pathway mechanism was responsible. For example, certain mutants of *Klebsiella pneumoniae* are killed as a result of immunoglobulin-independent, direct C1q binding to outer membrane porins and subsequent C′ activation (1, 2). However, in our experiments, killing was abolished by IgM depletion and restored by purified IgM addition, ruling out this possibility. Another mechanism of antibody-independent classical pathway activation is mediated by PC (40). In *H. influenzae*, PC present on certain types of LOS binds CRP, leading to C1q binding and subsequent killing (42). We were able to exclude this possibility, since depletion of CRP had no effect on the killing of FX517 and yet CRP-depleted sera no longer killed PC-expressing *H. influenzae*. Furthermore, we were unable to demonstrate a PC epitope on *H. ducreyi* LOS. Other mutants of gram-negative bacteria can activate the alternative pathway, but it was inferred that this did not occur, since inhibition of the classical/mannan binding protein pathway (with EGTA/Mg) abolished killing of FX517.

Negative regulators of complement. In order to better understand the serum resistance observed in *H. ducreyi*, we studied the direct binding of negative regulators of complement to *H. ducreyi*. Previously, we showed that vitronectin was bound by *H. ducreyi* expressing *dsrA*, but in the present study we found that vitronectin alone could not account for all of the observed serum resistance. Vitronectin inhibits the membrane attack complex, but differences in deposition of earlier complement components between 35000HP and FX517 were observed, suggesting vitronectin was not the sole mediator of serum resistance. In looking for earlier negative regulators of complement, we found about twofold more C4bp was bound by strain 35000HP than by FX517. Gonococcal control strain 6564 bound up to 60-fold more than strain 35000HP. Binding of C4bp would result in less C4 detected and later components on 35000HP than by FX517. Although this is what we observed, we also found that little C1q was bound by 35000HP prior to the step C4bp affects. Less C1q binding is consistent with decreased binding of IgM to 35000HP. Thus, the very modest C4bp binding cannot alone account for all of the differences in complement binding observed. It is possible that C4bp or vitronectin bound to 35000HP contributes partially to serum resistance in *H. ducreyi* 35000HP, but further studies are necessary. Surprisingly, *H. ducreyi* strain 35000HP is also very resistant to killing by a variety of specific surface-reactive antisera (19, 21). It is a distinct possibility that such antibodies initiate the classical pathway but that negative regulators of complement vitronectin or C4bp inhibit subsequent complement-mediated killing. Further studies are under way to determine if this occurs.

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